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This information is current as of May 8, 2016.

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*J Immunol* 2015; 195:194-202; Prepublished online 29 May 2015;

doi: 10.4049/jimmunol.1401892

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# Deficiency of CD40 Reveals an Important Role for LIGHT in Anti-*Leishmania* Immunity

Ifeoma Okwor,<sup>\*</sup> Guilian Xu,<sup>†</sup> Haidong Tang,<sup>‡</sup> Yong Liang,<sup>§</sup> Yang-Xin Fu,<sup>‡</sup> and Jude E. Uzonna<sup>\*,¶</sup>

We previously showed that LIGHT and its receptor herpes virus entry mediator (HVEM) are important for development of optimal CD4<sup>+</sup> Th1 cell immunity and resistance to primary *Leishmania major* infection in mice. In this study, we further characterized the contributions of this molecule in dendritic cell (DC) maturation, initiation, and maintenance of primary immunity and secondary anti-*Leishmania* immunity. Flow-cytometric studies showed that CD8 $\alpha$ <sup>+</sup> DC subset was mostly affected by HVEM-Ig and lymphotoxin  $\beta$  receptor-Ig treatment. LIGHT signaling is required at both the priming and the maintenance stages of primary anti-*Leishmania* immunity but is completely dispensable during secondary immunity in wild type mice. However, LIGHT blockade led to impaired IL-12 and IFN- $\gamma$  responses and loss of resistance in healed CD40-deficient mice after *L. major* challenge. The protective effect of LIGHT was mediated primarily via its interaction with lymphotoxin  $\beta$  receptor on CD8 $\alpha$ <sup>+</sup> DCs. Collectively, our results show that although LIGHT is critical for maintenance of primary Th1 response, it is dispensable during secondary anti-*Leishmania* immunity in the presence of functional CD40 signaling as seen in wild type mice. *The Journal of Immunology*, 2015, 195: 194–202.

The outcome of infection with the intracellular pathogen *Leishmania major*, the causative agent of cutaneous leishmaniasis, is dependent on the nature of CD4<sup>+</sup> Th cell subset that is induced (1–4). Resistance is usually correlated with the development of strong IFN- $\gamma$ -producing CD4<sup>+</sup> Th1 cells, which activate macrophages to produce NO, an effector molecule for killing intracellular parasites. In contrast, susceptibility has been associated with IL-4 and IL-10 production by Th2 cells, cytokines that deactivate macrophages and inhibit their intracellular parasite-killing ability (1, 5). A key factor that regulates the nature and quality of anti-*Leishmania* immunity in infected mice is the level of IL-12 production by dendritic cells (DCs) and the degree of responsiveness of naive CD4<sup>+</sup> T cells to this cytokine (6–8). Thus, BALB/c mice are highly susceptible to *L. major* because their DCs produce very low amounts of IL-12, and their CD4<sup>+</sup> T cells respond poorly to it because of the downregulation of IL-12R $\beta$  expression (8–10). In contrast, DCs from the resistant C57BL/6 (B6) mice produce high levels of IL-12 and their

CD4<sup>+</sup> T cells maintain IL-12 responsiveness throughout infection (8). The pathways that influence early IL-12 production by DCs during *L. major* infection are still poorly understood, although the nature of activation and expression of various costimulatory molecules appear to be important (11, 12).

The TNF superfamily of cytokines and their receptors play critical roles in the development of the immune system and in immune regulation, inflammation, and autoimmunity by regulating cell death, survival, and differentiation (13–15). There are four closely related members of this family, which include lymphotoxin  $\alpha$  (LT $\alpha$ , LT $\beta$ , TNF, and LIGHT. Their cognate receptors include TNFR1, TNFR2, lymphotoxin  $\beta$  receptor (LT $\beta$ R), and herpes virus entry mediator (HVEM). Both LT $\alpha$  and LT $\beta$  are important for formation and structural integrity of peripheral lymphoid organs because mice with targeted deletion of these genes lack organized peripheral lymph nodes and their spleen architecture is structurally dysregulated (16–18). In contrast, LIGHT-deficient mice have normal peripheral lymphoid organs, suggesting that unlike LT $\alpha$  and LT $\beta$ , its function may be more closely related to immune modulation (16, 17). LIGHT is expressed on activated T cells, monocytes, granulocytes, and immature DCs (19–21) and binds to three receptors: LT $\beta$ R, HVEM, and in humans, the decoy receptor, DcR3/TR6 (19, 22, 23). HVEM is expressed on resting T cells, NK cells, monocytes, immature DCs, and endothelial cells (22, 24, 25), whereas LT $\beta$ R is expressed mostly on stromal cells, DCs, and endothelial cells but is absent on lymphocytes (26, 27). The interaction of LIGHT with its receptors modulates several biological processes including cell survival, inflammation, and upregulation of intracellular adhesion molecule-1 leading to tumor eradication (28). In addition, signals transmitted via LIGHT–HVEM interaction have costimulatory effects on T cells, enhancing their proliferation and cytokine production (19, 21). Thus, LIGHT expression enhances cytolytic T lymphocyte-mediated tumor immunity and allograft rejection (21, 29), and its overexpression on T cells results in extensive T cell-proliferative disorders that are characterized by massive polyclonal expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (30, 31). We previously reported that blockade of LIGHT signaling results in severe impairment in anti-CD40, LPS-, and CpG-induced IL-

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Received for publication July 24, 2014. Accepted for publication May 4, 2015.

This work was supported by the Canadian Institutes for Health Research (CIHR; to J.E.U.), the Manitoba Health Research Council (to J.E.U.), a CIHR-Frederic Banting and Charles Best Doctoral Award (to I.O.), and the CIHR-International Infectious Disease and Global Health Training Program (to I.O.).

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Abbreviations used in this article: B6, C57BL/6; BMDC, bone marrow-derived DC; DC, dendritic cell; dLN, draining lymph node; DTH, delayed-type hypersensitivity; HVEM, herpes virus entry mediator; KO, knockout; LT, lymphotoxin; LT $\beta$ R, LT  $\beta$  receptor; RANK, receptor activator of NF- $\kappa$ B; SLA, soluble leishmanial Ag; TRANCE, TNF-related activation-induced cytokine; WT, wild type.

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12p40 production by macrophages and DCs in vitro and in vivo, and a concomitant impairment in CD4<sup>+</sup> Th1 response and susceptibility to *L. major* in the otherwise resistant B6 mice (32). In this study, we extend these findings by showing that blockade of LIGHT interaction with its receptor leads to impaired DC maturation and IL-12 production post-*L. major* infection. We further demonstrate that LIGHT is required at both the priming and the maintenance stages of primary anti-*Leishmania* immunity but is completely dispensable during secondary immunity. Interestingly, blockade of LIGHT signaling in CD40-deficient (knockout [KO]) mice that healed their primary *L. major* infection after rIL-12 treatment led to lower delayed-type hypersensitivity (DTH) and higher parasite burden after secondary *L. major* challenge. Collectively, our results show that LIGHT is critical for priming and maintenance of CD4<sup>+</sup> Th1 response during primary *L. major* infection but is completely dispensable for secondary immunity in intact wild type (WT) mice. However, in the absence of CD40 signaling, LIGHT becomes important for sustained IL-12 production, recall CD4<sup>+</sup> Th1 response, and maintenance of secondary anti-*Leishmania* immunity.

## Materials and Methods

### Mice

Six- to 8-wk-old female B6 mice were purchased from Charles River Laboratories (St-Constant, QC). Female CD40-deficient (CD40 KO) and IL-12 KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free units at the Central Animal Care Services, University of Manitoba. All mouse experiments were approved by the University of Manitoba Animal Care Committee in accordance with the regulation of the Canadian Council for Animal Care.

### Parasites, infection, and rIL-12 treatment

*L. major* parasites (MHOM/IL/80/Friedlin) were grown in Grace's insect medium (Life Technologies Life Technologies) supplemented with 20% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. For infection, 7-d stationary-phase promastigotes were washed three times in PBS and counted. Mice were infected by injecting  $1 \times 10^6$  parasites suspended in 50 µl PBS into the right hind footpad. For challenge infections, healed mice (>12 wk postinfection) were injected with  $5 \times 10^6$  parasites suspended in 50 µl PBS into the left hind footpad. Lesion development and progression were monitored weekly by measuring the diameter of the infected and uninfected footpads with Vernier calipers. Infected WT mice completely resolved their lesions by 12 wk postinfection. To obtain healed CD40 KO mice, we treated infected CD40 KO and IL-12 KO mice intralesionally with 0.3 µg rIL-12 (PeproTech, Rocky Hill, NJ) three times per week for the first 2 wk of infection. This treatment results in complete lesion resolution by 8–10 wk after infection with *L. major*.

### Treatment with HVEM-Ig, LTβR-Ig, and TRANCE-Ig fusion proteins

One day before *L. major* infection, some naive mice were injected with HVEM-Ig or LTβR-Ig fusion proteins (100 µg/mouse) or control-Ig (Human IgG1; Sigma) i.p. (32). To determine whether LIGHT signaling is important at the initiation (priming) and/or maintenance of established immune response, we initiated HVEM-Ig administration 1 d before (as described earlier) or 2 wk post-*L. major* infection. To investigate the role of LIGHT in secondary (memory) anti-*Leishmania* immunity, we injected healed WT and CD40 KO mice with HVEM-Ig or LTβR-Ig (100 µg/mouse) i.p. and then challenged them with *L. major* on the contralateral footpad the next day. To investigate the role of TNF-related activation-induced cytokine (TRANCE) receptor activator of NF-κB (RANK) pathway in secondary anti-*Leishmania* immunity, we injected healed WT or CD40KO mice with TRANCE-Ig (100 µg/mouse) i.p. and then challenged them with *L. major* on the contralateral foot the following day. Challenged mice were further given weekly administration of the fusion protein until sacrificed. Mice were monitored for DTH response at 72 h postchallenge and sacrificed 3 wk later to estimate parasite burden.

### In vitro recall response and intracellular cytokine staining

At various times postinfection, infected mice were sacrificed and the draining lymph nodes (dLNs) were collected and made into single-cell suspensions. Cells were washed, suspended at 4 million/ml in complete medium (DMEM

supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin), and plated at 1 ml/well in 24-well tissue culture plates (Falcon; VWR, Edmonton, AB). Cells were stimulated with or without soluble leishmanial Ag (SLA; 50 µg/ml) for 72 h, and the supernatant fluids were collected and stored at –20°C until assayed for cytokines by ELISA. For intracellular cytokine staining, some cells were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 4 h, and brefeldin A (10 µg/ml) was added in the last 2 h to enhance intracellular cytokine accumulation. Cells were harvested and stained for cell-surface markers and intracellular cytokines as previously described (33).

### ELISA

IL-4, IL-12, and IFN-γ concentrations in the cell culture supernatant fluids were measured by sandwich ELISA using paired Abs from BD Pharmingen (San Jose, CA) according to manufacturer's suggested protocols. The sensitivities of the ELISAs were 15, 31, and 15 pg/ml for IL-4, IL-12, and IFN-γ, respectively.

### Isolation of dLN and splenic DCs

CD11c<sup>+</sup> cells were enriched from pooled dLNs (three mice per pool) of 3-d *L. major*-infected WT mice treated with HVEM-Ig, LTβR-Ig, or control Ig, or from spleens of naive WT or CD40 KO mice using Stem Cell EasySep enrichment kit following manufacturer's instructions. The enriched cells were then stained with allophycocyanin-conjugated anti-CD8α eFlour-450-conjugated anti-CD103 mAbs and then sorted into CD11c<sup>+</sup>CD8α<sup>+</sup>, CD11c<sup>+</sup>CD8α<sup>–</sup>, CD11c<sup>+</sup>CD103<sup>+</sup>, and CD11c<sup>+</sup>CD103<sup>–</sup> populations using BD FACSAria III (BD Biosciences, Mississauga, ON). The purity of the different cell population was >96% as assessed by flow cytometry.

### Generation of bone marrow-derived DCs

Bone marrow-derived DCs (BMDCs) were generated from naive WT, HVEM KO, and LTβR KO mice as described previously (34). In brief, bone marrow cells were isolated from the femur and tibia of mice. BMDCs were differentiated in petri dishes in the presence of 20 ng/ml rGM-CSF (PeproTech, Rocky Hill, NJ). Immature DCs were harvested on day 8, and the purity of DCs (i.e., CD11c<sup>+</sup> cells) was between 85 and 92% as assessed by flow cytometry.

### CD4<sup>+</sup> T cell purification and CFSE labeling

CD4<sup>+</sup> T cells were purified from single-cell suspensions of spleens from naive or *L. major*-infected and healed (>12 wk postinfection) WT or CD40 KO mice by negative selection using CD4<sup>+</sup> T cell enrichment kit (EasySep; Stemcell Technologies, Vancouver, BC) according to the manufacturer's suggested protocols. To maximize T cell recovery, we first labeled total spleen cells with CFSE dye as previously described (35) before CD4<sup>+</sup> T cell enrichment. The purity of T cells was >98% as assessed by flow cytometry.

### Coculture of DCs and T cells

Sorted splenic DCs or BMDCs were cocultured in U-bottom 96-well tissue culture plates with CFSE-labeled CD4<sup>+</sup> T cells at a DC/T cell ratio of 1:100. The cells were stimulated with or without freeze-thawed *L. major* Ag ( $5 \times 10^6$  parasite equivalent per well) for 4 d. Proliferation and IFN-γ secretion were then assessed by flow cytometry. In some experiments, the sorted DC subsets from the dLNs of fusion protein-treated and *L. major*-infected mice were stimulated in vitro with SLA for 3 d and the level of IL-12p40 in the culture supernatant fluid was assessed by ELISA.

### Estimation of parasite burden

Parasite burden in the primary infected or challenged footpads was quantified by limiting dilution analysis as previously described (33).

### Statistical analysis

Student *t* test was used to compare mean and SEM between two groups. In some experiments, nonparametric one-way ANOVA was used to compare mean and SD of more than two groups. Tukey's test was used where there was significant difference in ANOVA. Differences were considered significant when *p* < 0.05.

## Results

### Blockade of LIGHT leads to reduction in DC number and IL-12 production post-*L. major* infection

We showed previously that blockade of LIGHT by HVEM-Ig or LTβR-Ig affects LPS-induced production of IL-12 by BMDCs

in vitro (32). To test whether LIGHT blockade affected the subset of DCs that is induced post-*L. major* infection, we treated naive mice with HVEM-Ig, LT $\beta$ R-Ig, or control-Ig before infection with *L. major* and assessed the subset of DCs present in the dLNs directly ex vivo by flow cytometry. Blockade of LIGHT caused significant ( $p < 0.05$ – $0.01$ ) reduction in the percentages (Fig. 1A, 1C) and absolute numbers (Fig. 1B, 1D) of CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> (Fig. 1A, 1B) and CD11c<sup>+</sup>CD103<sup>+</sup> (Fig. 1C, 1D) DCs in the dLNs of infected mice. In addition, we found that both CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD103<sup>+</sup> DCs were capable of producing IL-12 (Fig. 1E, 1F). However, the CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> DCs subsets were the major population that produced IL-12 post-*L. major* infection (Fig. 1E, 1F). Interestingly, IL-12 production by CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> cells was significantly ( $p < 0.01$ – $0.001$ ) reduced post-HVEM-Ig and -LT $\beta$ R-Ig treatment (Fig. 1E), whereas only LT $\beta$ R-Ig fusion protein caused significant reduction in IL-12 production in both CD103<sup>+</sup> and CD103<sup>-</sup> DC subsets (Fig. 1F). Taken together, these results show that LIGHT affected the numbers and subsets of DCs in the dLNs, as well as their ability to produce IL-12 after infection with *L. major*.

#### *LIGHT is important for both priming and maintenance of primary anti-Leishmania immunity*

LIGHT is important for optimal IL-12 production, the development of CD4<sup>+</sup> Th1 immune response, and resistance to *L. major* (32). Because IL-12 is required for both priming and maintenance of anti-*Leishmania* immunity (36–38), we investigated whether LIGHT is required at both the priming and the maintenance stages of anti-*Leishmania* immunity. Injection of HVEM-Ig (Fig. 2A, 2B) or LT $\beta$ R-Ig (data not shown) fusion proteins before or starting at 2 wk after *L. major* infection resulted in significantly ( $p < 0.001$ ) bigger lesion size and higher parasite burden than in the control-Ig-treated group (Fig. 2A, 2B). This treatment also severely impaired the production of IFN- $\gamma$  by CD4<sup>+</sup> T cells after in vitro stimulation with SLA (Fig. 2C–E). However, the effect of LIGHT blockade was more prominent in mice treated with the fusion protein pre-infection, suggesting that although this pathway is important for both effective priming and maintenance of anti-*Leishmania* immunity, it is more important during the initiation phase of the anti-*Leishmania* immune response.

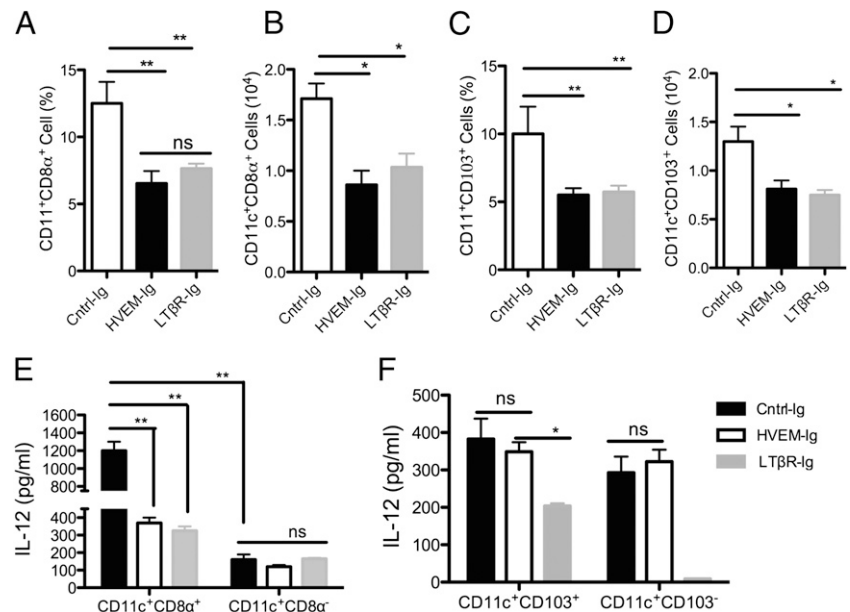
#### *LIGHT is dispensable for secondary anti-Leishmania immunity*

Next, we wished to determine whether LIGHT is also important for effective secondary (memory) anti-*Leishmania* immunity. Therefore, we injected HVEM-Ig or LT $\beta$ R-Ig into healed B6 mice (>16 wk after primary infection), and after 24 h challenged them with *L. major*. Injection of HVEM-Ig or LT $\beta$ R-Ig before secondary challenge had no effect on DTH response (Fig. 3A) and rapid control of parasite proliferation (Fig. 3B). Furthermore, whereas injection of HVEM-Ig or LT $\beta$ R-Ig significantly impaired IL-12 production by dLN cells from infected naive mice (see Figs. 1, 2), it had no effect on the IL-12 production by cells from healed mice after secondary *L. major* challenge (Fig. 3C). Taken together, these results suggest that although LIGHT is critical for effective primary immunity, it is dispensable during secondary anti-*Leishmania* immunity.

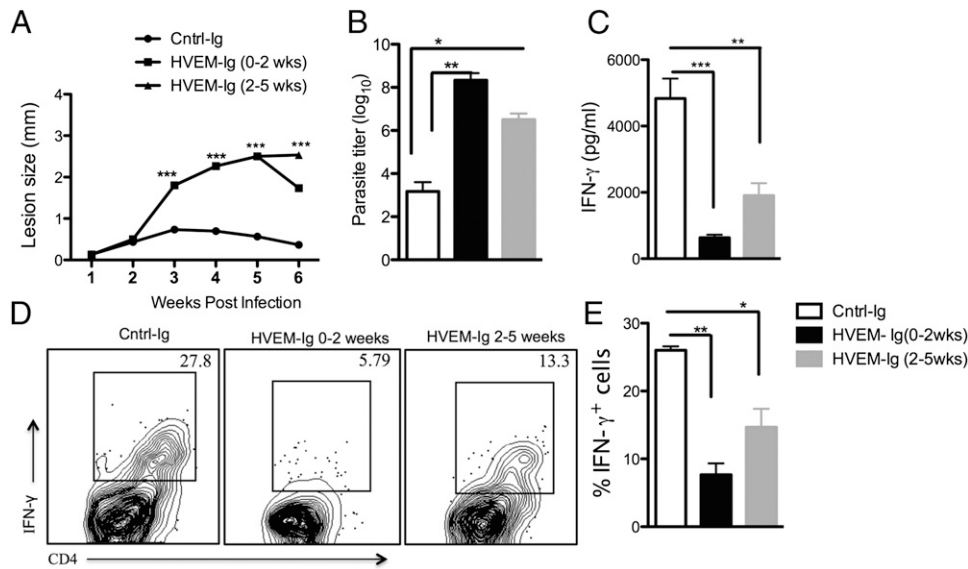
#### *Treatment with rIL-12 leads to healing in CD40-deficient mice*

Although an earlier report showed that CD40 and CD40L interaction is critical for resistance to *L. major* (39), a later study showed that this interaction may be dispensable for healing low-dose infection (40). Because IL-12 production is indispensable for resistance to *L. major*, this observation suggests the existence of an alternative (i.e., CD40-CD40L-independent) pathway for IL-12 production during *L. major* infection. Indeed, the outcome of *L. major* infection in CD40- and IL-12-deficient mice after treatment with rIL-12 was completely different. Although treatment of both mice with rIL-12 within the first 2 wk of infection initially led to resolution of cutaneous lesion and reduction in parasite burden, lesion recrudescence (indicative of disease reactivation) occurred in IL-12 KO, but not in CD40 KO, mice after cessation of rIL-12 treatment (Fig. 4A, 4B). Furthermore, although rIL-12-treated CD40 KO mice were resistant to secondary *L. major* challenge, treated IL-12 KO mice were highly susceptible (Fig. 4C). These observations underscore the critical role of CD40-CD40L interaction in IL-12 production and immunity against *L. major* infection. More importantly, they further confirm the existence of a CD40-CD40L-independent, IL-12-producing pathway for maintenance of secondary immunity in healed CD40 KO mice.

**FIGURE 1.** Blockade of LIGHT leads to reduction in numbers of CD8 $\alpha$ <sup>+</sup> and CD103<sup>+</sup> DCs and IL-12 production post-*L. major* infection. B6 mice were treated with HVEM-Ig, LT $\beta$ R-Ig, or control-Ig and infected with *L. major* the next day. Three days postinfection, mice were sacrificed and the percentages (A and C) and absolute numbers (B and D) of CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> (A and B) and CD11c<sup>+</sup>CD103<sup>+</sup> (C and D) cells in the lymph nodes draining the infection site were determined directly ex vivo by flow cytometry. DCs (CD11c<sup>+</sup>) were first enriched by negative selection from pooled dLN cells from three mice and then sorted into CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD8 $\alpha$ <sup>-</sup> (E) or CD11c<sup>+</sup>CD103<sup>+</sup> and CD11c<sup>+</sup>CD103<sup>-</sup> (F) populations. The cells were then stimulated in vitro with soluble *Leishmania* Ag (SLA; 50  $\mu$ g/ml) for 3 d in the presence or absence of HVEM-Ig, LT $\beta$ R-Ig, or control-Ig, and the level of IL-12 in the culture supernatant fluid was determined by ELISA. Results presented are representative of two different experiments with similar results ( $n = 4$ – $5$  mice/group). \* $p < 0.05$ , \*\* $p < 0.01$ . ns, not significant.







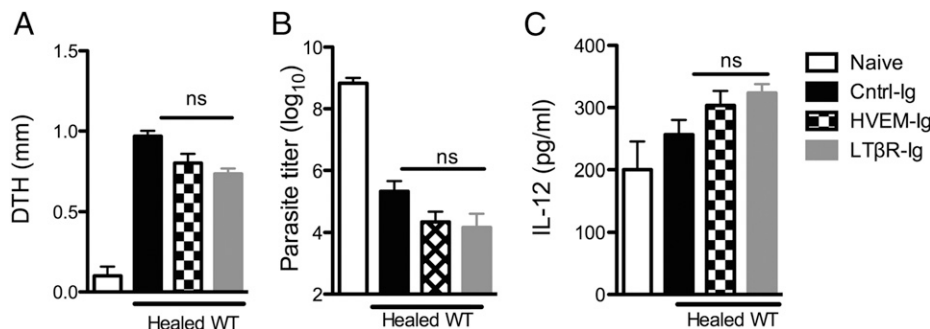
**FIGURE 2.** LIGHT is important for both priming and maintenance of anti-*Leishmania* immunity. B6 mice were injected with HVEM-Ig or control-Ig weekly starting at day -1 for 2 wk (to test priming) or from 2 wk after (to test maintenance) infection with *L. major*, and the lesion size (**A**) was monitored weekly. Six weeks postinfection, mice were sacrificed to estimate parasite burden in the infected footpads (**B**). At sacrifice, the dLN cells were restimulated in vitro for 72 h with SLA and the supernatant fluids were assayed for IFN-γ by ELISA (**C**). In addition, the percentages of IFN-γ-producing CD4<sup>+</sup> T cells were determined by flow cytometry (**D**). The bar graph (**E**) represents the mean ± SE of flow-cytometry results (shown in **C**) from four to five mice in each group. Results presented are representative of two different experiments with similar results ( $n = 4-5$  mice/group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### LIGHT is critical for maintenance of immunity in healed CD40-deficient mice

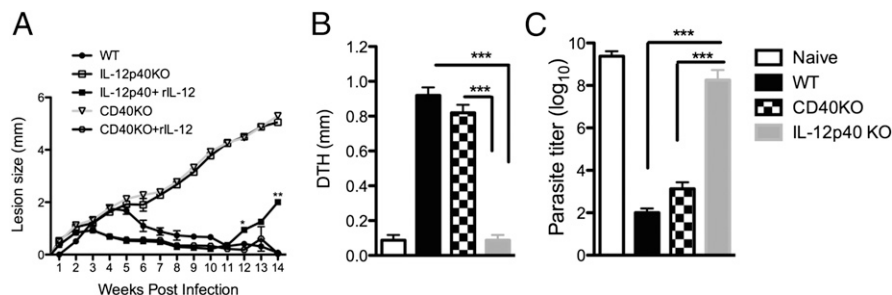
Our findings implicate LIGHT as playing an important role in IL-12 production and optimal resistance to *L. major* infection (Figs. 1–3) (32). Because healed CD40 KO mice do not reactivate their lesion (unlike IL-12 KO mice) after cessation of rIL-12 treatment, we hypothesized that their durable resistance is due to LIGHT-dependent IL-12 production. We therefore assessed the impact of HVEM-Ig or LTβR-Ig treatment on secondary anti-*Leishmania* immunity in rIL-12-treated CD40 KO mice. Unlike WT mice, treatment of healed CD40 KO mice with HVEM-Ig or LTβR-Ig led to loss of immunity to secondary *L. major* challenge as evidenced by significantly ( $p < 0.01$ ) smaller DTH response and higher parasite burden at the challenged footpads (Fig. 5A, 5B). In addition, dLN cells from healed CD40 KO mice treated with HVEM-Ig or LTβR-Ig produced significantly

( $p < 0.01-0.001$ ) less IL-12 (Fig. 5C) and IFN-γ (Fig. 5D) compared with the control-Ig-treated group. Furthermore, the level of IL-4 (Fig. 5E) was significantly ( $p < 0.01$ ) higher in the HVEM-Ig- or LTβR-Ig-treated group compared with the control-Ig-treated group.

A previous report by Padigel et al. (41) showed that in CD40L KO mice, the TRANCE-RANK pathway mediates IL-12 production leading to resistance to *L. major*. Therefore, we investigated whether the TRANCE-RANK pathway played a role in the maintenance of resistance in IL-12-treated CD40 KO mice infected with *L. major*. We found that blockade of TRANCE-RANK pathway by treatment with TRANCE-Ig in healed CD40 KO mice had no impact on DTH response and rapid parasite control after secondary *L. major* challenge (Fig. 6A, 6B). Collectively, these results indicate that in the absence of CD40, LIGHT plays a critical role in IL-12 production, long-term maintenance of immunity, and resistance to secondary *L. major* challenge.



**FIGURE 3.** LIGHT is dispensable for secondary (memory) anti-*Leishmania* immunity. B6 mice were infected with *L. major* and allowed to heal. Twelve weeks after primary infection (when lesion was completely resolved), healed mice were injected with HVEM-Ig, LTβR-Ig, or control-Ig (100 μg/mouse) and challenged the next day in the contralateral feet with *L. major*. Some healed or uninfected age-matched (naive) WT mice were also challenged as controls. Three days after challenge, DTH response was measured using calipers (**A**). The healed mice further received weekly injections of fusion proteins (or control Ig) and at 3 wk postchallenge, they were sacrificed to determine parasite burden (**B**). The dLN cells were stimulated in vitro with SLA for 72 h and the supernatant fluids were assayed for IL-12p40 by ELISA (**C**). Results presented are representative of three different experiments with similar results ( $n = 3-5$  mice/group). ns, not significant.



**FIGURE 4.** Treatment with rIL-12 leads to healing in *L. major*-infected CD40 KO, but not IL-12 KO mice. WT, IL-12 KO, and CD40 KO mice infected with *L. major* were treated with rIL-12 or PBS for the first 2 wk and lesion development (A) was monitored weekly. At 12 wk postinfection, mice were challenged with virulent *L. major*, and DTH response was determined after 72 h. (B) Challenged mice were sacrificed after 3 wk, and parasite burden in the challenged feet was determined by limiting dilution assay (C). Results presented are representative of three independent experiments with similar results ( $n = 3-4$  mice/group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

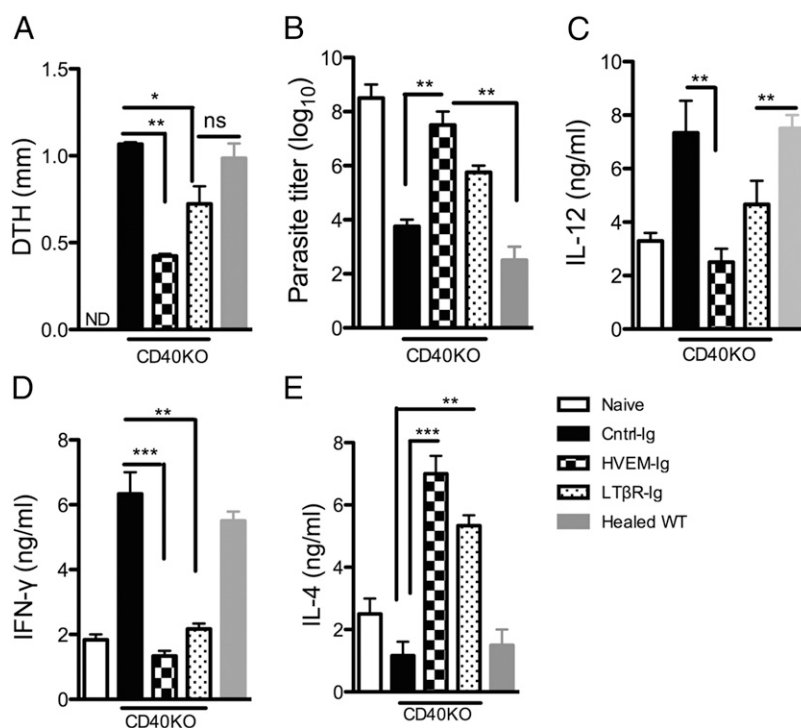
#### CD8 $\alpha^+$ DCs are responsible for CD4 $^+$ T cell proliferation and IFN- $\gamma$ production in healed CD40 KO mice

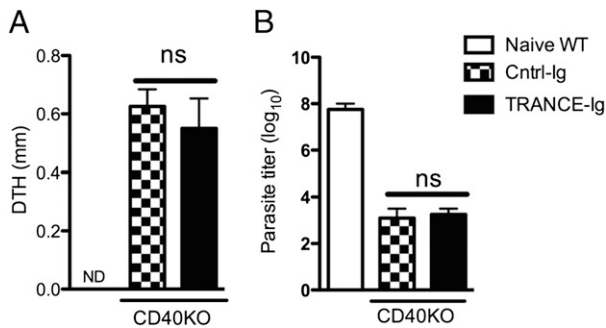
Next, we wished to determine the relevant DC population that drives CD40-independent but IL-12-dependent maintenance of anti-*Leishmania* immunity in healed CD40 KO mice. We sorted splenic DCs (CD11c $^+$ ) from WT and CD40 KO mice into highly enriched CD8 $\alpha^+$  (CD11c $^+$ CD8 $\alpha^+$ ) or CD8 $\alpha^-$  (CD11c $^+$ CD8 $\alpha^-$ ) and CD103 $^+$  (CD11c $^+$ CD103 $^+$ ) and CD103 $^-$  (CD11c $^+$ CD103 $^-$ ) populations, pulsed them with freeze-thawed *L. major*, and cocultured them with CFSE-labeled CD4 $^+$  T cells from healed WT mice, and assessed T cell proliferation and IFN- $\gamma$  production after 4 d. We found that both CD8 $\alpha^+$  and CD8 $\alpha^-$  DCs from WT mice induced comparable proliferation and IFN- $\gamma$  production by CD4 $^+$  T cells (Fig. 7A, upper panel, and 7B). In contrast, only CD8 $\alpha^+$  DCs from CD40 KO mice induced appreciable proliferation and IFN- $\gamma$  production by CD4 $^+$  T cells from WT mice (Fig. 7A, lower panel, and 7B). Interestingly, CD103 $^+$  and CD103 $^-$  DCs from WT and CD40 KO mice induced comparable numbers of proliferating and IFN- $\gamma$ -producing CD4 $^+$  T cells in vitro (Fig. 7C, 7D). Taken together, these results suggest that CD8 $\alpha^+$  DCs are responsible for maintaining CD40-independent CD4 $^+$  T cell response and immunity in healed CD40 KO mice.

#### LIGHT mediates IL-12 production and induction of CD4 $^+$ T cell proliferation and IFN- $\gamma$ production primarily via LT $\beta$ R

In mice, LIGHT interacts with HVEM and LT $\beta$ R, two major receptors expressed on APCs including DCs (19, 22, 23). To determine the key receptor involved in LIGHT-mediated IL-12 production and induction of CD4 $^+$  T cell response, we cocultured *L. major*-infected BMDCs from WT, HVEM KO, and LT $\beta$ R KO with CFSE-labeled CD4 $^+$  T cells from healed WT mice, and assessed cell proliferation and IFN- $\gamma$  production after 4 d. As shown in Fig. 8A, IL-12 production was significantly ( $p < 0.001$ ) reduced in cell-culture supernatants of CD4 $^+$  T cells cocultured with LT $\beta$ R KO BMDCs compared with those cocultured with WT or HVEM KO BMDCs. Interestingly, the level of IL-12 was significantly ( $p < 0.01$ ) lower in the LT $\beta$ R KO compared with the HVEM KO mice (Fig. 8A). In line with this, the percentage of proliferating IFN- $\gamma$ -producing CD4 $^+$  T cells in conditions cocultured with LT $\beta$ R KO BMDCs was significantly ( $p < 0.01-0.001$ ) lower than those cocultured with WT or HVEM KO BMDCs (Fig. 8B, 8C). Taken together, our data suggest that although binding of LIGHT to HVEM may play some roles, LT $\beta$ R is the primary receptor involved in LIGHT-mediated IL-12 production, Th1 response, and immunity to *L. major*.

**FIGURE 5.** Blockade of LIGHT led to loss of immunity in healed CD40 KO mice. CD40 KO mice made to heal their *L. major* infection by rIL-12 treatment were treated with HVEM-Ig, LT $\beta$ R-Ig, or control-Ig and then challenged with *L. major* after 24 h. Some uninfected age-matched (naive) mice were also challenged as controls. Seventy-two hours after challenge, DTH response (A) was determined by measuring footpad thickness in the challenge feet. Three weeks after challenge, mice were sacrificed and parasite burden in the challenged footpads was determined (B). At sacrifice, dLN cells were stimulated with SLA for 72 h, and the levels of IL-12 (C), IFN- $\gamma$  (D), and IL-4 (E) in the cell culture supernatant fluids were determined by ELISA. Results presented are representative of two independent experiments with similar findings ( $n = 3-4$  mice/group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .





**FIGURE 6.** TRANCE–RANK interaction is not required for secondary immunity in healed CD40 KO mice. Healed CD40 KO mice were treated with TRANCE-Ig fusion protein or control-Ig 24 h before challenge with *L. major*, and DTH response was determined after 72 h (A). Three weeks after challenge, mice were sacrificed and parasite burden in the challenged footpads was determined (B). Results presented are representative of two independent experiments with similar findings. ns, not significant.

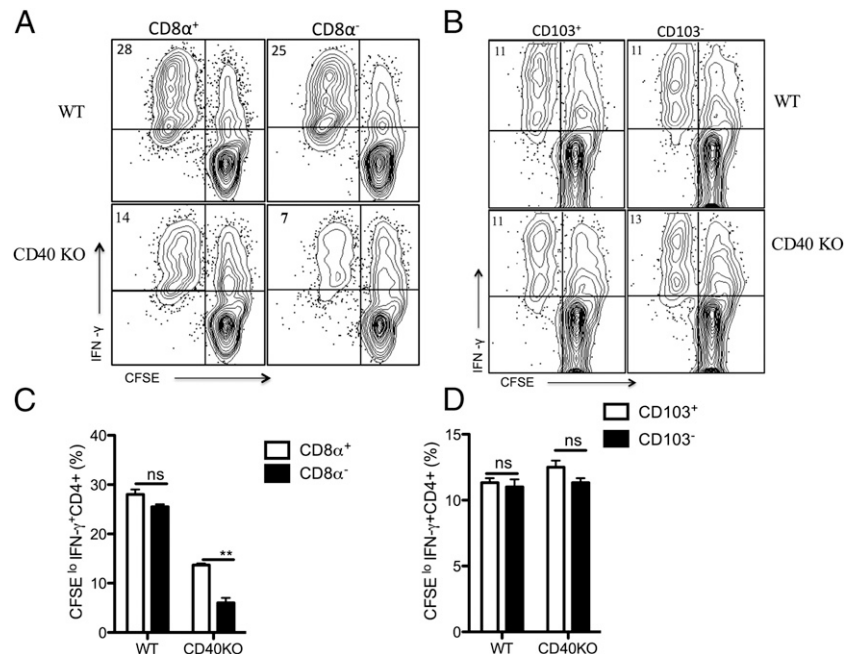
## Discussion

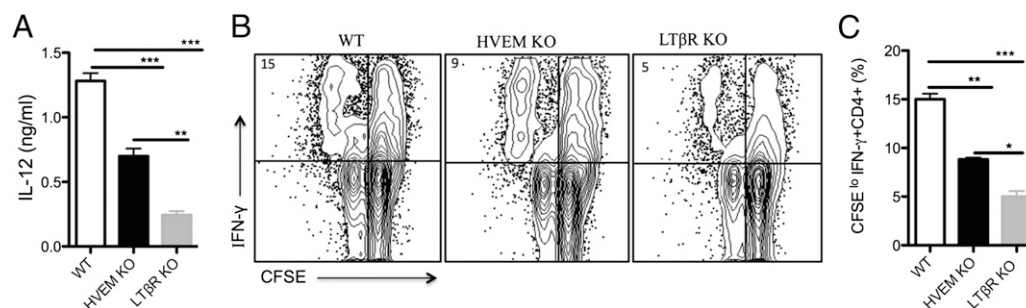
We previously reported that LIGHT signaling is critical for optimal IL-12 production and CD4<sup>+</sup> Th1 development in mice, and its blockade either by using HVEM-Ig fusion protein or targeted gene deletion of LIGHT or HVEM results in susceptibility to *L. major* infection in the otherwise resistant strain of mice (32). However, this study did not directly show the contribution of LIGHT in IL-12 production post-*L. major* infection or investigate whether LIGHT is important for early and/or sustained IL-12 production in *L. major*-infected mice. Furthermore, whether LIGHT is required for secondary anti-*Leishmania* immunity was not investigated. In this study, we corroborate the importance of LIGHT in primary anti-*Leishmania* immunity. More importantly, we show that LIGHT is required at both the priming and the maintenance stages of primary anti-*Leishmania* immunity but is completely dispensable during secondary anti-*Leishmania* immunity in intact (WT) mice with functional CD40–CD40L interaction. However, in the absence of CD40–CD40L interaction (as is seen in CD40 KO mice), blockade of LIGHT led to dramatically impaired DTH and IFN- $\gamma$  responses and a consequent inability to control parasite

proliferation after secondary *L. major* challenge. We further found that CD8 $\alpha$ <sup>+</sup> DCs are responsible for maintaining this CD40-independent but IL-12-dependent CD4<sup>+</sup> T cell proliferation and IFN- $\gamma$  production in *L. major*-infected CD40 KO mice manipulated to heal their primary infection by rIL-12 treatment. Collectively, this study identifies LIGHT as an important molecule that is critical for maintenance of IL-12-dependent anti-*Leishmania* immunity in mice.

LIGHT is expressed on activated T cells, monocytes, granulocytes, and immature DCs (19–21) and binds to three receptors: LT $\beta$ R, HVEM, and in humans, the decoy receptor, DcR3/TR6 (19, 22, 23). HVEM is expressed on resting T cells, NK cells, monocytes, immature DCs, and endothelial cells (22, 24, 25), whereas LT $\beta$ R is expressed mostly on stromal cells, DCs, and endothelial cells but is absent on lymphocytes (26, 27). The interaction of LIGHT with its receptors including LT $\beta$ R, HVEM, and in humans, the decoy receptor, DcR3/TR6 (19, 22, 23), modulates several biological processes including cell survival, inflammation, and upregulation of intracellular adhesion molecule-1 leading to tumor eradication (28). In addition, signals transmitted via LIGHT–HVEM interaction have costimulatory effects on T cells, enhancing their proliferation and cytokine production (19, 21). Thus, LIGHT expression enhances cytolytic T lymphocyte-mediated tumor immunity and allograft rejection (21, 29), and its overexpression on T cells results in extensive T cell-proliferative disorders that are characterized by massive polyclonal expansion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells (30, 31). Several studies show that the engagement of LIGHT with its ligand plays a crucial role for optimal T cell activation and differentiation into effector cells (21, 22, 42, 43). Thus, blockade of LIGHT–HVEM interaction in vitro inhibits polyclonal T cell proliferation (22, 42), and T cells from LIGHT-deficient mice show impaired effector functions after anti-CD3 stimulation in vitro (44, 45). In addition to having a direct effect on T cells, LIGHT also indirectly influences activation and differentiation, and effector T cell responses by inducing DC maturation and IL-12 production (46, 47). We found that blockade of LIGHT by treatment with HVEM-Ig or LT $\beta$ R-Ig significantly impairs expression of costimulatory molecules and IL-12 production by total CD11c<sup>+</sup> cells (data not shown), as well as CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup> and CD11c<sup>+</sup>CD103<sup>+</sup> (Fig. 1)

**FIGURE 7.** CD8 $\alpha$ <sup>+</sup> DCs are responsible for maintaining CD40-independent CD4<sup>+</sup> T cell response in healed mice. CD11c<sup>+</sup> cells were enriched from spleens of naive WT or CD40 KO mice by positive selection and then further sorted by flow cytometry into CD11c<sup>+</sup>CD8 $\alpha$ <sup>+</sup>, CD11c<sup>+</sup>CD8 $\alpha$ <sup>−</sup>, CD11c<sup>+</sup>CD103<sup>+</sup>, and CD11c<sup>+</sup>CD103<sup>−</sup> populations. The different DC populations were then cocultured with CFSE-labeled CD4<sup>+</sup> T cells isolated from spleens of WT mice that have healed their primary *L. major* infection. After 96 h, the percentage of proliferating and IFN- $\gamma$ -producing CD4<sup>+</sup> T cells induced by the different subsets was determined by flow cytometry (A and B). The bar graphs (C and D) represent the mean  $\pm$  SE of flow-cytometry results (shown in A and B) from three to four mice in each group. Results presented are representative of two independent experiments with similar findings. \*\**p* < 0.01. ns, not significant.





**FIGURE 8.** LTβR is the primary receptor involved in LIGHT-dependent maintenance of CD4<sup>+</sup> T cell response in healed mice. BMDCs were generated from naive WT, HVEM KO, and LTβR KO mice and then cocultured with CFSE-labeled CD4<sup>+</sup> T cells from mice that healed their primary *L. major* infection. The cultures were stimulated with freeze-thawed *Leishmania* parasites for 96 h, and the level of IL-12 in the cell culture supernatant fluid was determined by ELISA (A). In addition, the percentage of proliferating (CFSE<sup>lo</sup>) IFN-γ-producing CD4<sup>+</sup> T cells was determined by flow cytometry (B). (C) Bar graph represents the mean ± SE of proliferating (CFSE<sup>lo</sup>) IFN-γ-producing CD4<sup>+</sup> T cells from three mice in each group. Results presented are representative of two independent experiments with similar findings. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

DCs in the dLNs, leading to impairment of Th1 response post-*L. major* infection. This is in agreement with a recent publication that showed that LIGHT–HVEM interaction is critical for IL-12-, TNF-, and IFN-γ-producing T cells that are responsible for controlling hepatic infection in *L. donovani*-infected mice (48). Taken together, our results suggest that the interaction of LIGHT with its ligands on APCs (such as DCs) is an important pathway for LIGHT-mediated IL-12 production, T cell activation, and resistance to *L. major* in mice. Previously published data showed that injection of LTβR agonistic Ab in mice with established *L. donovani* infection led to protective immune response and parasite clearance, suggesting that LTβR is the key ligand involved in LIGHT-mediated anti-*Leishmania* immunity (48). Using BMDCs from mice lacking HVEM or LTβR, we corroborate this finding and further show that LIGHT interaction with LTβR was more critical than its interaction with HVEM in mediating a protective immune response because BMDCs from LTβR KO mice induced significantly less proliferation and IFN-γ production in CD4<sup>+</sup> T cells compared with BMDCs from HVEM KO mice.

IL-12 is a critical cytokine for the development and maintenance of Th1 cells and resistance to *L. major* (6, 7, 49). Hence we investigated at what stage during *Leishmania* infection that LIGHT is required for immunity. Treatment of WT mice with HVEM-Ig or LTβR-Ig before (priming/initiation of immunity) or after 2 wk (maintenance of immunity) of infection resulted in significantly bigger lesions and higher parasite burden than in untreated controls (Fig. 2A, 2B). This was associated with severely impaired production of IFN-γ by cells from spleens and dLNs of infected mice (Fig. 2C). Collectively, these observations suggest that akin to rIL-12, LIGHT is required at both the initiation and the maintenance stages of anti-*Leishmania* immunity. Interestingly, we found that treatment with HVEM-Ig and LTβR-Ig had no effect on IL-12 production, induction of DTH response, and accelerated parasite clearance (Fig. 3). This indicates that although LIGHT contributes to optimal development of primary anti-*Leishmania* immunity, it has no effect during secondary (memory) immunity to *L. major*. It is likely that the requirements for activating/recalling memory T cells are different from those needed for differentiation of naive cells into effector cells, with the latter being more dependent on LIGHT-mediated IL-12 production than the former. Indeed, previous reports show that the requirements for activating memory cells are relatively less stringent than those for naive cells, with memory cells requiring lesser costimulation, Ag, TCR-peptide avidity, and cytokines than naive cells (50, 51).

Treatment of infected IL-12-deficient mice with rIL-12 leads to resolution of cutaneous lesion and control of parasite proliferation

(37, 38). However, recrudescence occurs after cessation of rIL-12 treatment, suggesting that continuous IL-12 is important for initiation and maintenance of resistance in *L. major*-infected mice (38). We found that unlike IL-12-deficient mice, treatment of CD40-deficient mice with rIL-12 leads to resistance that is maintained indefinitely even after cessation of recombinant cytokine treatment (Fig. 4). This suggests the existence of a CD40-independent IL-12-producing pathway during the maintenance phase of anti-*Leishmania* immunity. We found that blockade of LIGHT in CD40-deficient mice that healed their lesion led to impaired DTH response and parasite clearance after secondary *L. major* challenge that was associated with significantly reduced IFN-γ response. Collectively, these observations show that the maintenance of resistance in *L. major*-infected, CD40-deficient mice after cessation of rIL-12 treatment is mediated by LIGHT-dependent IL-12 production. Thus, in the absence of CD40 signaling, LIGHT plays a critical role for IL-12 production, a finding that explains the differences in outcome of *L. major* infection in IL-12- and CD40-deficient mice after cessation of rIL-12 treatment.

A previous report showed that signaling via the TRANCE-RANK pathway could compensate for CD40–CD40L interaction in the production of IL-12 during primary *L. major* infection (41). However, whether the TRANCE-RANK pathway contributes to IL-12 production during secondary anti-*Leishmania* immunity is not known. We found that blockade of TRANCE-RANK interaction did not affect IL-12 production and resistance to secondary *L. major* challenge in CD40-deficient mice, suggesting that this pathway is not responsible for the maintenance of resistance to *L. major* in CD40-deficient mice after cessation of rIL-12 treatment. Collectively, the data presented in this article suggest that in the absence of CD40, IL-12 production via LIGHT pathway is enough to maintain secondary protective immunity against *L. major*.

In summary, we have demonstrated that LIGHT is important for DC maturation and IL-12 production after infection with *L. major*, and disruption of this pathway leads to impairment in CD4<sup>+</sup> Th1 development and IFN-γ production. Furthermore, we show that although LIGHT is important for the maintenance of anti-*Leishmania* immunity, it is dispensable for secondary (memory) anti-*Leishmania* immunity in WT mice. However, in the absence of CD40–CD40L signaling, LIGHT-dependent IL-12 production, primarily via CD8α<sup>+</sup> DCs, is critical for maintenance anti-*Leishmania* immunity. Our data corroborate our initial report and further suggest that LIGHT could provide a new therapeutic target for regulation of IL-12 production in vivo and for controlling various CD4<sup>+</sup> Th1-mediated autoimmune and inflammatory diseases such as arthritis, diabetes, colitis, among others.



## Acknowledgments

We thank the members of Parasite Vaccine Development Laboratory for insightful comments and constructive criticisms.

## Disclosures

The authors have no financial conflicts of interest.

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